BD GenomeWalker™ Universal Kit User Manual

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I. Introduction

BD GenomeWalker™ DNA walking is a simple method for finding unknown genomic DNA sequences adjacent to a known sequence such as a cDNA (Siebert *et al.*, 1995). BD GenomeWalker Kits (Cat. Nos. 638901, 638902, and 638903) are available for human, mouse, and rat genomes, respectively; however, researchers who are interested in other species need a more general approach. The **BD GenomeWalker™ Universal Kit** is designed with this in mind, enabling researchers to apply this powerful method of DNA walking to the species of their choice.

Using your genomic DNA of interest, the first step is to construct pools of uncloned, adaptor-ligated genomic DNA fragments, which are referred to for convenience as BD GenomeWalker "libraries." The starting DNA must be very clean and have a high average molecular weight, requiring a higher quality preparation than the minimum suitable for Southern blotting or conventional PCR. To ensure that your genomic DNA is of adequate quality, the kit includes controls for comparison. Separate aliquots of DNA are completely digested with different restriction enzymes that leave blunt ends. The BD GenomeWalker Universal Kit comes with a set of four restriction enzymes; however, alternative restriction enzymes that leave blunt ends may be substituted. Each batch of digested genomic DNA is then ligated separately to the BD GenomeWalker Adaptor.

After the libraries have been constructed, the protocol takes just two days and consists of two PCR amplifications per library (Figure 1). The first or primary PCR uses the outer adaptor primer (AP1) provided in the kit and an outer, gene-specific primer (GSP1) provided by the researcher. The primary PCR mixture is then diluted and used as a template for a secondary or "nested" PCR with the nested adaptor primer (AP2) and a nested gene-specific primer (GSP2). This generally produces a single, major PCR product from at least three of the four libraries (and often in all four; Figure 1). Each of the DNA fragments—which begin in a known sequence at the 5' end of GSP2 and extend into the unknown adjacent genomic DNA—can then be cloned and further analyzed.

The kit also provides human genomic DNA to be used as a positive control for library construction, as well as a preconstructed BD GenomeWalker human library as a positive control for PCR. Figure 2 shows typical results of primary and secondary PCR with these positive controls. Amplification of the *Pvu* II BD GenomeWalker human library with the adaptor primers and primers derived from exon 1 of the human tissue-type plasminogen activator (tPA) gene (PCP1 and PCP2) should generate a single major product 1.5 kb in length.

Long-distance PCR with the BD Advantage™ 2 PCR Kit

BD GenomeWalker reactions should be performed with a 50X polymerase mix containing a combination of DNA polymerases suitable for long-distance PCR (LD PCR; Barnes, 1994; Cheng *et al.*, 1994). In LD PCR, a combination of two thermostable DNA polymerases is used to increase the range and accuracy of

I. Introduction *continued*

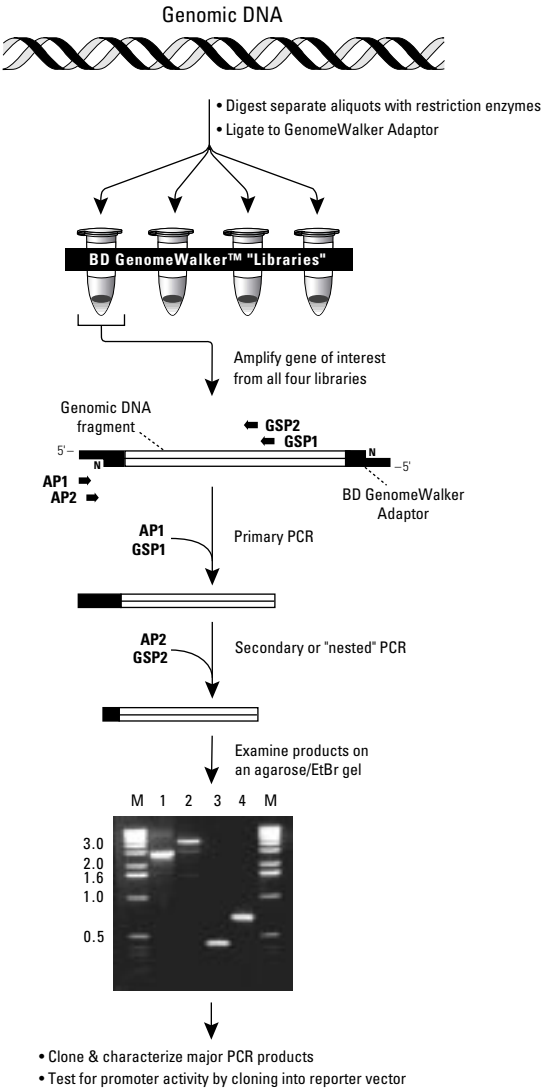

Figure 1. Flow chart of the BD GenomeWalker™ protocol. The gel shows a typical result generated by walking with BD GenomeWalker human libraries and gene-specific primers. Lane 1: *Eco*R V Library. Lane 2: *Dra* I Library. Lane 3: *Pvu* II Library. Lane 4: *Ssp* I Library. Lane M: DNA size markers. The absence of a major product in one of the libraries is not unusual. In our experience, there is no major band in one or more lanes in approximately half of the BD GenomeWalker experiments. As explained in the Expected Results and Troubleshooting Guide (Section VI), this is usually because the distance between the primer and the upstream restriction site is greater than the capability of the system. N: Amine group that blocks extension of the 3' end of the adaptor-ligated genomic fragments. AP: Adaptor primers. GSP: Gene-specific primers.

I. Introduction *continued*

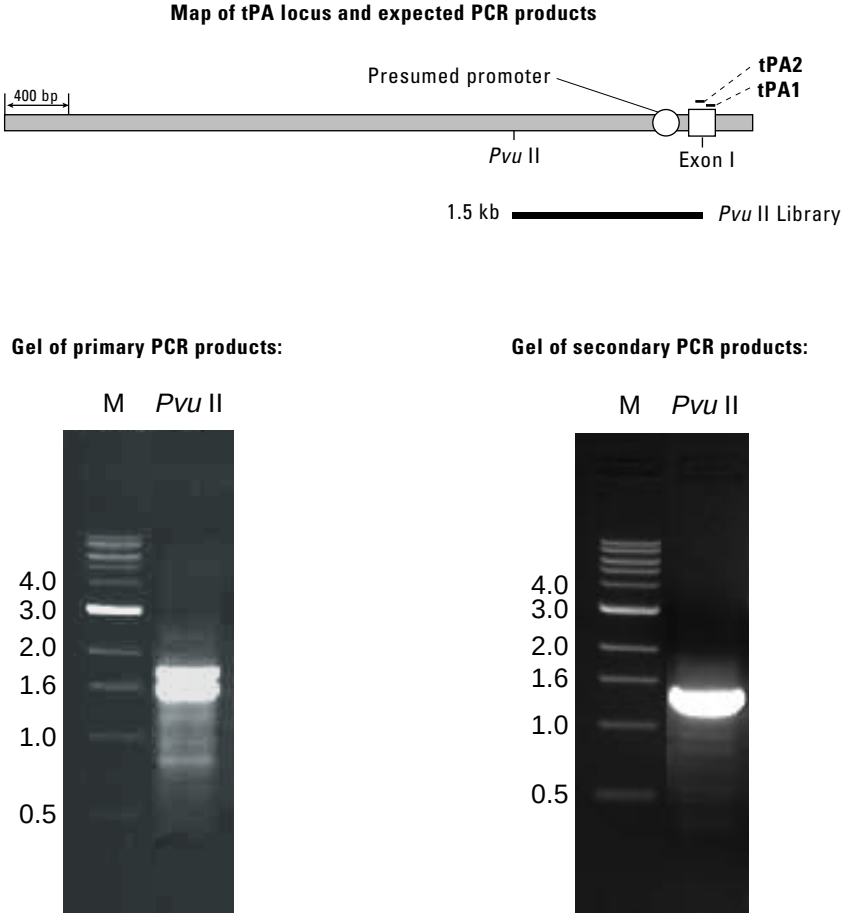

Figure 2. Map of the human tissue-type plasminogen activator (tPA) locus (Friezner-Degen *et al.,* 1986) and results of primary and secondary BD GenomeWalker™ PCR using tPA primers. Primary and secondary (nested) PCR was performed using BD Advantage 2 Polymerase Mix and the cycling parameters described in the protocol. The tPA primers used in this experiment are the positive control primers PCP1 and PCP2 provided with the kit. Lane M: 1 kb ladder of DNA size markers.

I. Introduction *continued*

PCR amplification. Most of the extension is carried out by a primary polymerase, while a secondary polymerase provides the critical 3'→5' exonuclease or "editing" function that corrects misincorporated nucleotides.

In the BD GenomeWalker protocol, the use of LD PCR extends the range of possible PCR products to about 6 kb. (The precise reason for the upper limit on BD GenomeWalker products is not clear. It may be due to the loss of the suppression PCR effect [see Appendix B]). As discussed in Section III, we recommend our BD Advantage™ 2 Polymerase Mix (Cat. No. 639201). BD Advantage 2 Polymerase Mix is available separately and in the BD Advantage™ 2 PCR Kit (Cat. No. 639206).

Applications

The BD GenomeWalker Universal Kit enables researchers to create uncloned libraries for walking by PCR in any genomic DNA. In less than a week, the method provides access to the genomic DNA sequences adjacent to a known DNA sequence in any species. Using both the BD SMART RACE™ cDNA Amplification Kit (Cat. No. 634914) and the BD GenomeWalker Universal Kit, you can clone full-length cDNAs and the surrounding genomic sequences without ever screening a library. In addition to obtaining promoters, BD GenomeWalker DNA walking can also be used to map intron/exon junctions and to walk bidirectionally from any sequence-tagged site (STS) or expressed sequence tag (EST). Although individual steps are limited to about 6 kb, multiple steps can be strung together to create longer walks. Consequently, this method is useful for filling in gaps in genome maps, particularly when the missing clones have been difficult to obtain by conventional library screening methods. In all applications, BD GenomeWalker PCR products are generally pure enough to allow restriction mapping without cloning. Nevertheless, a discussion of cloning PCR products and testing them for promoter activity is included at the end of this manual.

II. List of Components

Store human genomic DNA at 4°C; all other components at –20°C.

Note: These reagents are sufficient for constructing three sets of four BD GenomeWalker libraries. Each construction is enough for 80 reactions.

- **Restriction enzymes and buffers:**
  - 30 µl *Dra* I (10 units/µl)
  - 100 µl 10X *Dra* I Restriction Buffer
  - 25 µl *Eco*R V (10 units/µl)
  - 50 µl 10X *Eco*R V Restriction Buffer
  - 50 µl *Pvu* II (10 units/µl)
  - 100 µl 10X *Pvu* II Restriction Buffer
  - 25 µl *Stu* I (10 units/µl)
  - 50 µl 10X *Stu* I Restriction Buffer
- 75 µl **Control Human Genomic DNA** (0.1 µg/µl)
- 10 µl **T4 DNA Ligase** (6 units/µl)
- 40 µl **10X Ligation Buffer**
- 36 µl **BD GenomeWalker Adaptor** (25 µM)
- 250 µl **Adaptor Primer 1** (AP1; 10 µM)
- 250 µl **Nested Adaptor Primer 2** (AP2; 10 µM)
  See Figure 3 on the next page for the sequences of AP1 & AP2.
- 10 µl **BD GenomeWalker Human Positive Control Library**
- 50 µl **Positive Control tPA Primer** (PCP1; 10 µM)
- 50 µl **Positive Control tPA Nested Primer** (PCP2; 10 µM)
  See Appendix A for the sequences of the positive control primers supplied with the kit.

II. List of Components *continued*

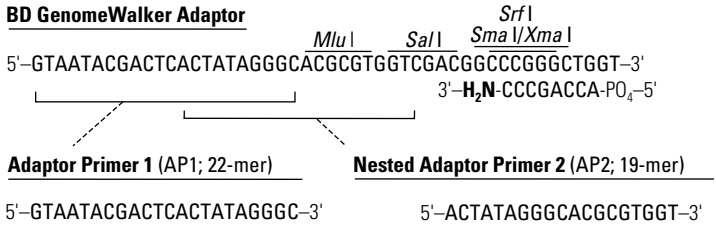

Figure 3. Structure of the BD GenomeWalker™ adaptor and adaptor primers. The adaptor is ligated to both ends of the genomic DNA fragments to create BD GenomeWalker libraries. The amine group on the lower strand of the adaptor blocks extension of the 3' end of the adaptor-ligated genomic fragments, and thus prevents formation of an AP1 binding site on the general population of fragments. The design of the adaptor and adaptor primers is critical for the suppression PCR effect (Figure 5). The T_m's of AP1 and AP2 are 59°C and 71°C, determined by nearest neighbor analysis (Freier *et al.*, 1986).

III. Additional Materials Required

The following reagents are required but not supplied:

- **Phenol**
- **Chloroform**
- **Glycogen** (10 µg/µl)
- **3 M sodium acetate**
- **95% ethanol**
- **80% ethanol**
- **TE** 10 mM Tris, 0.1 mM EDTA (10/0.1, pH 7.5)
- **TE** 10 mM Tris, 1 mM EDTA (10/1, pH 7.5)
- **0.5X TBE buffer or TAE buffer** (See Note in Section VII.B.1)
- **BD Advantage 2 Polymerase Mix** (50X)
  You will need a *Taq*-based 50X polymerase mix suitable for LD PCR. Conventional PCR with a single polymerase will not produce a band in most BD GenomeWalker experiments. This protocol has been optimized with the BD Advantage 2 Polymerase Mix (Cat. No. 639201). This enzyme mix was specifically developed for PCR amplifications of genomic DNA templates of all sizes. This 50X mix contains BD TITANIUM™ *Taq* DNA Polymerase—a nuclease-deficient N-terminal deletion of *Taq* DNA polymerase plus BD TaqStart™ Antibody to provide automatic hot start PCR (Kellogg et al., 1994)—and a minor amount of a proofreading polymerase. BD Advantage 2 Polymerase Mix is also available in the BD Advantage 2 PCR Kit (Cat. No. 639206).

III. Additional Materials Required *continued*

- **BD TaqStart™ Antibody** (Cat. No. 639250)

  If you are not using BD Advantage 2 Polymerase Mix, we strongly recommend that you use some form of hot start in BD GenomeWalker PCR. To do this, simply include BD TaqStart Antibody in the 50X polymerase mix (see PT1576-1, available at **www.bdbiosciences.com/clontech**). BD TaqStart Antibody is included in BD Advantage 2 Polymerase Mix. This antibody is an effective method for hot start PCR that is simpler and more convenient than wax-based or manual methods. The BD TaqStart Antibody binds to and inactivates *Taq* DNA polymerase and thus eliminates DNA synthesis from nonspecifically bound primers while reactions are being assembled. PCR amplification proceeds efficiently after an initial 1 min incubation at 94°C, which irreversibly inactivates the BD TaqStart Antibody. See Kellogg *et al.* (1994) for a discussion of hot start PCR with inactivating antibodies. Hot start with wax beads (Chou *et al.*, 1992) or manual hot start (D'aquila *et al.*, 1991) can also be used.
- **10X PCR reaction buffer:** If you are using a DNA polymerase mix other than BD Advantage 2 Polymerase Mix, use the PCR reaction buffer provided with the enzyme mix.
- **dNTP mix:** 10 mM each of dATP, dCTP, dGTP & dTTP. Store at –20°C.
- **0.5-ml PCR tubes**
  We recommend GeneAmp 0.5-ml PCR Reaction Tubes
  (PE Biosystems, Cat. No. N801-0737 or N801-0180.)
- **Deionized H_2O** (Milli-Q™-filtered or equivalent)
- **1 kb ladder of DNA size markers**

The following product is not required but recommended:

- BD Advantage™ 2 PCR Kit (Cat. No. 639206 or 639207)

30 rxns	100 rxns	
30 µl	100 µl	50X BD Advantage 2 Polymerase Mix
200 µl	600 µl	10X BD Advantage 2 PCR Buffer
200 µl	600 µl	10X BD Advantage 2 SA PCR Buffer
50 µl	120 µl	50X dNTP Mix (10 mM each)
30 µl	100 µl	Control DNA Template (100 ng/µl)
30 µl	100 µl	Control Primer Mix (10 µM)
2.5 ml	5.0 ml	PCR-Grade Water
		User Manual (PT3281-1)
		Protocol-at-a-Glance (PT3281-2)

IV. Construction of BD GenomeWalker™ Libraries

A. General Considerations

1. Construction of BD GenomeWalker DNA libraries should begin with very clean, high-molecular weight genomic DNA. This requires a higher quality preparation than the minimum suitable for Southern blotting or conventional PCR. Isolation procedures for genomic DNA can be found in various reference manuals (e.g., Ausubel *et al.*, 1994; Sambrook *et al.*,1987); however, keep in mind that methods vary for different species. To ensure that your genomic DNA is of adequate quality, follow the procedure described in Section IV.B.
2. Work in an area away from all PCR products. Use only equipment that is not exposed to PCR products.
3. For PCR, use only deionized H_2O (Milli-Q or equivalent). Do not use DEPC-treated or autoclaved H_2O.
4. Human genomic DNA and positive control gene-specific primers (PCP1 and PCP2) are provided to test the system. They are designed to walk upstream from exon I of the human tissue-type plasminogen activator gene.
5. The following protocol is designed for the construction of four libraries from experimental genomic DNA and one *Pvu* II library from positive control human genomic DNA (provided in kit).

B. Quality of Genomic DNA

1. Check the size of genomic DNA on a 0.5% agarose/EtBr gel.

   Load 1 µl of experimental genomic DNA (0.1 µg/µl) and 1 µl of control genomic DNA (0.1 µg/µl) on a 0.5% agarose/EtBr gel in 0.5X TBE, along with DNA size markers, such as a 1 kb ladder or λ/*Hin*d III digest. Genomic DNA should be bigger than 50 kb with minimum smearing.
2. Check the purity of genomic DNA by *Dra* I digestion.
   a. In a 0.5-ml reaction tube, combine the following:

5	µl	Experimental genomic DNA
1.6	µl	*Dra* I (10 units/µl)
2	µl	10X *Dra* I Restriction Buffer
11.4	µl	Deionized H_2O

   Also set up a control digestion without enzyme.

   b. Mix gently by inverting tube. Do not vortex; vigorous mixing will shear genomic DNA.

   c. Incubate at 37°C overnight.

   d. Run 5 µl of each reaction on a 0.5% agarose/EtBr gel along with 0.5 µl of experimental genomic DNA as a control. At this point, you should see a smear, indicating that your DNA can be digested by restriction enzymes.

IV. Construction of BD GenomeWalker™ Libraries *continued*

C. Digestion of Genomic DNA

For each library construction, you should set up a total of five reactions. For your experimental genomic DNA, set up four blunt-end digestions—one for each blunt-end restriction enzyme provided. Additionally, set up one *Pvu* II digestion of human genomic DNA as a positive control.

1. Label five 1.5-ml tubes: DL1, DL2, DL3, DL4, and positive control.
2. For each reaction, combine the following in a separate 1.5-ml tube:

25	µl	Genomic DNA (0.1 µg/µl)
8	µl	Restriction enzyme (10 units/µl)
10	µl	Restriction enzyme buffer (10X)
57	µl	Deionized H_2O

   Mix gently by inverting tube. Do not vortex. Vigorous mixing will shear genomic DNA.
3. Incubate at 37°C for 2 hr.
4. Vortex the reaction at slow speed for 5–10 sec. Return to 37°C overnight (16–18 hr).
5. From each reaction tube, remove 5 µl and run on a 0.5% agarose/EtBr gel to determine whether digestion is complete. You may wish to save an additional aliquot of each sample to run on the gel used in Step D.17 (see below).

D. Purification of DNA

1. To each reaction tube, add an equal volume (95 µl) of phenol.
2. Vortex at slow speed for 5–10 sec.
3. Spin briefly to separate the aqueous and organic phases.
4. Using a pipet, transfer the upper (aqueous) layer into a fresh 1.5-ml tube. Discard the lower (organic) layer properly into the chlorinated hazardous waste.
5. To each tube, add an equal volume (95 µl) of chloroform.
6. Vortex at slow speed for 5–10 sec.
7. Spin briefly to separate the aqueous and organic phases.
8. Using a pipet, transfer the upper (aqueous) layer into a fresh 1.5-ml tube. Discard the lower (organic) layer properly into the chlorinated hazardous waste.
9. To each tube, add 2 volumes (190 µl) of ice cold 95% ethanol, 1/10 volume (9.5 µl) of 3 M NaOAc (pH 4.5), and 20 µg of glycogen.
10. Vortex at slow speed for 5–10 sec.
11. Centrifuge at 15,000 rpm for 10 min.
12. Decant supernatant and wash pellet in 100 µl of ice cold 80% ethanol.

IV. Construction of BD GenomeWalker™ Libraries *continued*

13. Centrifuge at 15,000 rpm for 5 min.
14. Decant supernatant and air dry the pellet.
15. Dissolve pellet in 20 µl of TE (10/0.1, pH 7.5).
16. Vortex at slow speed for 5–10 sec.
17. From each reaction tube, remove 1 µl and run on a 0.5% agarose/EtBr gel to determine the approximate quantity of DNA after purification.

E. Ligation of Genomic DNA to BD GenomeWalker™ Adaptors

For each library construction, you should set up a total of five ligation reactions. You will have four blunt-end digestions of your experimental genomic DNA and one positive control *Pvu* II digestion of human genomic DNA.

1. From each tube, transfer 4 µl of digested, purified DNA to a fresh 0.5-ml tube. To each, add the following:

   1.9 µl BD GenomeWalker Adaptor (25 µM)
   1.6 µl 10X Ligation Buffer
   0.5 µl T4 DNA Ligase (6 units/µl)

2. Incubate at 16°C overnight.

   Note: A PCR thermal cycler holds a very constant temperature and is recommended in place of a water bath for this reaction.

3. To stop the reactions, incubate at 70°C for 5 min.
4. To each tube, add 72 µl of TE (10/1, pH 7.5).
5. Vortex at slow speed for 10–15 sec.

V. BD GenomeWalker™ DNA Walking

A. Primer Design

You will need to design two gene-specific primers—one for primary PCR (GSP1) and one for secondary PCR (GSP2). The nested PCR primer should anneal to sequences beyond the 3' end of the primary PCR primer (i.e., upstream of the primary PCR primer when walking upstream and downstream of the primary PCR primer when walking downstream). Whenever possible, the outer and nested primers should not overlap; if overlapping primers must be used, the 3' end of the nested primer should have as much unique sequence as possible.

In general, the gene-specific primers should be derived from sequences as close to the end of the known sequence as possible. For walking upstream from cDNA sequence, the primer should be as close to the 5' end as possible. Ideally, the primers should be derived from the first exon of the gene. If primers are derived from downstream exons, the resulting PCR products are less likely to contain the promoter, particularly if the intervening intron(s) and exon(s) comprise more than a few kb (see Figure 2).

Gene-specific primers should be 26–30 nucleotides in length and have a GC content of 40–60%. (Even if the T_m's seem high, do not design primers shorter than 26 bp. At BD Biosciences Clontech, we typically use 27-mers.) This will ensure that the primers will effectively anneal to the template at the recommended annealing and extension temperature of 67°C. Primers should not be able to fold back and form intramolecular hydrogen bonds, and sequences at the 3' end of your primers should not be able to anneal to the 3' end of the adaptor primers. There should be no more than three G's and C's in the last six positions at the 3' end of the primer.

Five restriction sites have been incorporated into the BD GenomeWalker Adaptor—*Sal* I (cohesive ends), *Mlu* I (cohesive ends), and overlapping *Srf* I (cohesive ends), *Sma* I (blunt ends), and *Xma* I (cohesive ends) sites. The sites in the Adaptor Primer allow easy insertion of PCR products into commonly used promoter reporter vectors. If you wish to use other restriction sites to clone the resulting PCR products, suitable sites should also be designed into the 5' end of GSP2 (i.e., the nested gene-specific primer used for secondary PCR.) Alternatively, BD GenomeWalker PCR products can be cloned into a general purpose cloning vector using restriction sites, or into a TA-type cloning vector using the A overhang left by *Taq* DNA polymerase. (See Section VII.B.3 for a discussion of our various promoter-cloning reporter vectors and reporter assay systems.)

B. General Considerations

1. Cycling parameters

   The cycling parameters in this protocol have been optimized using the PE Biosystems DNA Thermal Cycler 480, BD Advantage™ 2 Polymerase Mix, and the reagents and positive control primers provided in

V. BD GenomeWalker™ DNA Walking *continued*

the BD GenomeWalker Kit. The optimal cycling parameters may vary with different polymerase mixes, gene-specific primers, and thermal cyclers. Recommended cycling parameters for the PE Biosystems GeneAmp PCR Systems 2400 and 9600 are provided in Appendix C. Please refer to the Troubleshooting Guide (Section VI) for suggestions on optimizing PCR conditions.

2. Use some form of hot start PCR

   It is advantageous to use some form of hot start in PCR, and the protocol assumes that BD TaqStart™ Antibody has been included in the 50X polymerase mix (see Section III, Additional Materials). Hot start can also be performed using wax beads (Chou *et al.*, 1992) or manually (D'Aquila *et al.*, 1991). If you use a manual or wax-based hot start, you will need to adapt the protocol to these particular methods.

3. Touchdown PCR

   The PCR cycling parameters in steps V.C.9 and V.C.18 are for "touchdown" PCR (Don *et al.*, 1991; Roux, 1995; Hecker and Roux, 1996). Touchdown PCR involves using an annealing/extension temperature that is several degrees *higher* than the T_m of the primers during the initial PCR cycles. Although primer annealing (and amplification) is less efficient at this higher temperature, it is much more specific. The higher temperature also enhances the suppression PCR effect with AP1 (see Appendix B), allowing a critical amount of gene-specific product to accumulate. The annealing/extension temperature is then reduced to slightly below the primer T_m for the remaining PCR cycles, permitting efficient, exponential amplification of the gene-specific product. As noted above, we recommend using primers with T_m's greater than 68°C to allow you to use the touchdown cycling programs given in this protocol.

4. Use of the positive controls

   In each experiment, we suggest that you include a positive control in which you amplify the supplied control library using the positive control primers (PCP1 and PCP2). This will confirm that your DNA polymerase mix is functional and thermal cycling parameters are compatible with the BD GenomeWalker protocol.

5. Amplify all four libraries with each set of GSPs

   To maximize your chances of success, we recommend that you amplify all four libraries with each new gene-specific primer.

6. Use the recommended amounts of enzymes

   The enzyme amounts have been carefully optimized for the BD GenomeWalker amplification protocol and reagents.

V. BD GenomeWalker™ DNA Walking *continued*

C. Procedure for PCR-based DNA Walking in BD GenomeWalker Libraries

The BD GenomeWalker DNA walking protocol consists of eight primary and secondary PCR amplifications: four experimental libraries, two positive controls (BD GenomeWalker Human Positive Control Library and one positive control library constructed from Control Human Genomic DNA), and two negative controls (without templates). For both positive controls, use the positive control gene-specific primers, PCP1 and PCP2 (provided). For primary PCR, use 1 µl of each library. For secondary PCR, use 1 µl of a 50X dilution of the primary PCR product.

All BD GenomeWalker PCR steps have been optimized with the BD Advantage 2 Polymerase Mix, which includes BD TaqStart Antibody for automatic hot start PCR.

1. Label the 0.5-ml PCR tubes. For convenience, we suggest using the plan in Table I (GSP1 and GSP2 indicate your gene-specific primers):

TABLE I. SUGGESTED LABELING PLAN

DNA Library (DL)	Tube No.	1° PCR Primers	Tube No.	2° PCR Primers
DL-1	1A	GSP1 & AP1[a]	1B	GSP2 & AP2[b]
DL-2	2A	"	2B	"
DL-3	3A	"	3B	"
DL-4	4A	"	4B	"
Negative control No.1				
None	5A	"	5B	"
Positive control No.1				
Control library[c]	6A	PCP1 & AP1[a]	6B	PCP2 & AP2[b]
Negative control No.2				
None	7A	"	7B	"
Positive control No.2				
Pre-constructed[d] control library	8A	"	8B	"

[a] Primer contained in primary PCR master mix.
[b] Primer contained in secondary PCR master mix.
[c] Positive control for library construction. You construct this library from the control human genomic DNA provided in the kit (see Section IV).
[d] Positive control for PCR. This preconstructed library is included in the kit.

V. BD GenomeWalker™ DNA Walking *continued*

2. Prepare enough **primary PCR master mix** for all eight reactions plus one additional tube. Combine the following reagents in an 0.5-ml tube:

9 rxns	per rxn	
360 µl	40 µl	deionized H_2O
45 µl	5 µl	10X BD Advantage 2 PCR Buffer
9 µl	1 µl	dNTP (10 mM each)
9 µl	1 µl	AP1 (10 µM)
9 µl	1 µl	BD Advantage 2 Polymerase Mix (50X)
432 µl	48 µl	Total volume

   Mix well by vortexing (without introducing bubbles) and briefly spin the tube in a microcentrifuge.

3. Add 48 µl of the primary PCR master mix to the appropriately labeled tubes.
4. For reactions 1A through 5A, add 1 µl of GSP1 to each tube. For reactions 6A through 8A, add 1 µl of PCP1 to each tube.
5. Add 1 µl of each DNA library (including the positive control library) to the appropriately labeled tubes. **Do not add any library DNA to the negative control**.
6. Add 1 µl of H_2O to each negative control.
7. Overlay the contents of each tube with one drop of mineral oil and place caps firmly on tubes.
8. Briefly spin tubes in a microcentrifuge.
9. Commence cycling in a DNA Thermal Cycler 480 (PE Biosystems), using the following two-step cycle parameters:

   • 7 cycles:
   94°C 25 sec
   72°C 3 min

   • 32 cycles:
   94°C 25 sec
   67°C 3 min

   • 67°C for an additional 7 min after the final cycle.

   Note: Do not use a three-step cycling program (e.g., 95°C melting, 60°C annealing, 68°C extension). See Appendix C for cycling parameters for GeneAmp PCR Systems 2400 and 9600.

V. BD GenomeWalker™ DNA Walking *continued*

10. Analyze 5 µl of the primary PCR products on a 1.5% agarose/EtBr gel, along with DNA size markers such as a 1 kb ladder. If you do not see any product, perform five additional cycles.

Expected results of primary PCR: In all lanes except for negative controls, you should observe your predicted banding patterns. Be aware, however, that there may be smearing in some lanes, and you may observe a multiple banding pattern, ranging in size from about 500 bp to 5 kb. See Figure 2 in the Introduction (Section I) for a sample gel showing products of primary PCR. If you obtain any bands or smearing with your gene-specific primer, continue with secondary PCR as described in Steps 11–19 (even if your products are weaker than the positive control or the bands in Figure 2).

If you do not observe **any** product or smear with your gene-specific primers, consult the Troubleshooting Guide (Section VI).

11. Using a clean 0.5-ml tube for each sample, dilute 1 µl of each primary PCR (including positive and negative controls) into 49 µl of deionized H_2O.
12. Prepare a **secondary PCR master mix** for all eight reactions plus one additional tube. Combine the following reagents in an 0.5-ml tube:

9 rxns	per rxn	
360 µl	40 µl	deionized H_2O
45 µl	5 µl	10X BD Advantage 2 PCR buffer
9 µl	1 µl	dNTP (10 mM each)
9 µl	1 µl	AP2 (10 µM)
9 µl	1 µl	BD Advantage 2 Polymerase Mix (50X)
432 µl	48 µl	Total volume

Mix well by vortexing (without introducing bubbles) and briefly spin the tube in a microcentrifuge.

13. Add 48 µl of the secondary PCR master mix to the appropriately labeled tubes (Table I).
14. For reactions 1B through 5B, add 1 µl of GSP2 to each tube. For reactions 6B through 8B, add 1 µl of PCP2 to each tube.
15. Add 1 µl of each diluted primary PCR product (from Step 11) to the appropriately labeled tubes. Be sure to include the positive and negative controls.
16. Overlay the contents of each tube with one drop of mineral oil and place caps firmly on tubes.
17. Briefly spin tubes in a microcentrifuge.

V. BD GenomeWalker DNA Walking *continued*

18. Commence cycling in a DNA Thermal Cycler 480 (PE Biosystems), using the following two-step cycle parameters:

    • 5 cycles:
    94°C 25 sec
    72°C 3 min

    • 20 cycles:
    94°C 25 sec
    67°C 3 min

    • 67°C for an additional 7 min after the final cycle.

    Note: Do not use a three-step cycling program (e.g., 95°C melting, 60°C annealing, 68°C extension). See Appendix C for cycling parameters for the GeneAmp PCR Systems 2400 and 9600.

19. Analyze 5 µl of the secondary PCR products on a 1.5% agarose/EtBr gel, along with DNA size markers such as a 1 kb ladder or λ/*Hin*d III digest. If you do not see any product, perform four additional cycles.

    Store the unused portion of each secondary PCR at 4°C until you have confirmed that the procedure has been successful. At that point, proceed with analyzing and cloning the fragments of interest (e.g., putative promoter fragments), as described in Section VII.

VI. Expected Results and Troubleshooting Guide

A. Expected Results

1. Primary PCR

   A sample gel showing the results of primary BD GenomeWalker PCR can be seen in Figure 2 in the Introduction. In general, primary PCR should produce multiple fragments, ranging in size from about 500 bp to 5 kb. There may be smearing in some lanes. You should continue with secondary PCR if you obtain any bands or smearing with your gene-specific primer.

2. Secondary PCR

   a. Positive control primers (PCP1 and PCP2)

      The expected size of the band amplified from both the human positive control library and the library you constructed using the positive control human genomic DNA should be 1.5 kb.

   b. Experimental PCR primers

      In approximately half the cases, single major bands will be observed with each of the four libraries. The exact size of the major bands will depend on the positions of restriction sites in your gene. Typically, products of secondary PCR will range from 0.2 to 6 kb. Fragments generated from nested gene-specific primers that are less than 0.4 kb from one of the restriction sites represented in the BD GenomeWalker libraries may appear as a low molecular weight smear on a 1.5% agarose/EtBr gel. If this is the case with one or more of the BD GenomeWalker libraries, run the particular PCR product(s) on a 2% agarose/EtBr gel.

      In our experience, no product is observed in one or more of the libraries in approximately half the cases. This is usually because the distance from the primer to the restriction site is greater than the capability of the system (~6 kb). This limit reflects the diminished suppression PCR effect as template size increases. (For more information about suppression PCR, see Appendix B.) Targets greater than ~6 kb often become indistinguishable in a smear of high molecular weight material. Such smearing may also occur in lanes that do contain major bands, but should not affect the major bands. The absence of a major band in one or more of the libraries does not mean that products obtained with other libraries are not correct.

VI. Expected Results and Troubleshooting Guide *continued*

B. Troubleshooting Guide

1. No products with the positive control primers (even after increasing the number of primary cycles from 32 to 37)
   a. Reduce all annealing/extension temperatures by 2°C (i.e., 72°C to 70°C and 67°C to 65°C).
   b. Reduce the length of the incubation at 94°C.
   c. Check your 50X polymerase mix by PCR using two specific primers and a 1–10-kb template that has previously been successful.
2. Expected products observed with positive control primers, but no product observed either from library positive control or from your experimental libraries.
   a. Check the ligation step. If the PCR positive control produces the expected PCR product, but the control library and your experimental libraries do not, it is probably due to failure of your ligation. In this case, repeat the adaptor-DNA ligation step.
   b. Check the digestion and purification steps. The DNA concentration should be the same before and after phenol:chloroform extraction. Run samples of the DNA on an agarose gel before and after purification. If the intensity of EtBr staining is two-fold less after purification, you should concentrate the DNA. This can be accomplished either by ethanol precipitation or placing tubes in a rotating evaporator (e.g., Savant SpeedVac), and resuspending the DNA in a lower volume.
3. Expected products observed with positive control primers (for both the PCR positive control and the library control), but no product observed with your gene-specific primers
   a. Try decreasing the temperature for annealing and extension to 65°C or lower.
   b. Check the design of your primers. If the positive control PCP primers produce the expected PCR products, but your gene-specific primers do not produce major PCR products with any of the libraries, you will probably need to redesign your primers. If your primer sequence was derived from cDNA sequence information, the primary or secondary PCR primer may cross an exon/intron junction. If this is the case, it will be necessary to redesign one or both gene-specific primers. Remember that all primers should be able to anneal efficiently at 70°C (i.e., have a $T_m \geq 70°C$).

      If you are sure your primers do not cross intron/exon boundaries, recheck the sequence of your primers. In some instances, primers will fail to produce any products due to a mistake in primer design or synthesis.

VI. Expected Results and Troubleshooting Guide *continued*

c. Your target template may have a high GC-content. Such templates are difficult to amplify. Repeat your experiment using a final concentration of 5% DMSO in primary and secondary PCR. For each PCR, add 2.5 µl of DMSO (and only 37.5 µl of deionized H_2O) to the Master Mix (Step V.C.2). Add the DMSO to the Master Mix last. **Note:** You will need to perform more cycles in the presence of DMSO. For the primary PCR, perform 36 cycles instead of 32; for the secondary PCR, perform 24 cycles.

If this fails, repeat again using a final concentration of 6% DMSO and 3% glycerol in primary and secondary PCR.

If neither DMSO concentration solves the problem, try increasing the temperature to 99°C for five seconds at the beginning of the first cycle.

4. Nonspecific PCR products observed with your gene-specific primers.

Generally, the simplified touchdown PCR cycling program suggested in this protocol can significantly improve BD GenomeWalker results by increasing specificity. However, if you still observe nonspecific products, the following methods may help.

a. If possible, redesign your GSPs to have T_m's greater than 70°C. For this purpose, GSPs should be 26–30 bp in length, with a GC content of 40–60%. Do not design primers shorter than 26 bp.

b. If it is impossible to redesign your GSPs, try a touchdown PCR cycling program. For primary PCR, start with an annealing temperature of 72°C and decrease it by 1°C every second cycle to a "touchdown" at 67°C. Keep the annealing temperature at 67°C for the remaining 32 cycles. For secondary PCR, follow the same procedure, but use only 20 cycles after the annealing temperature reaches 67°C.

c. The problem may result from incomplete restriction digestion of your DNA. If this is the case, repeat to ensure that digestion is complete. Normally, if the DNA is completely digested, a single major band should be observed after secondary PCR. However, multiple bands may result from the species used (e.g., some plants are multiploid) or from genes that belong to multi-gene families.

VII. Suggestions for Characterizing BD GenomeWalker™ Products

A. Restriction Mapping of BD GenomeWalker PCR Products

BD GenomeWalker PCR products are generally clean enough to allow simple restriction mapping without cloning. An example of such an experiment is shown in Figure 4.

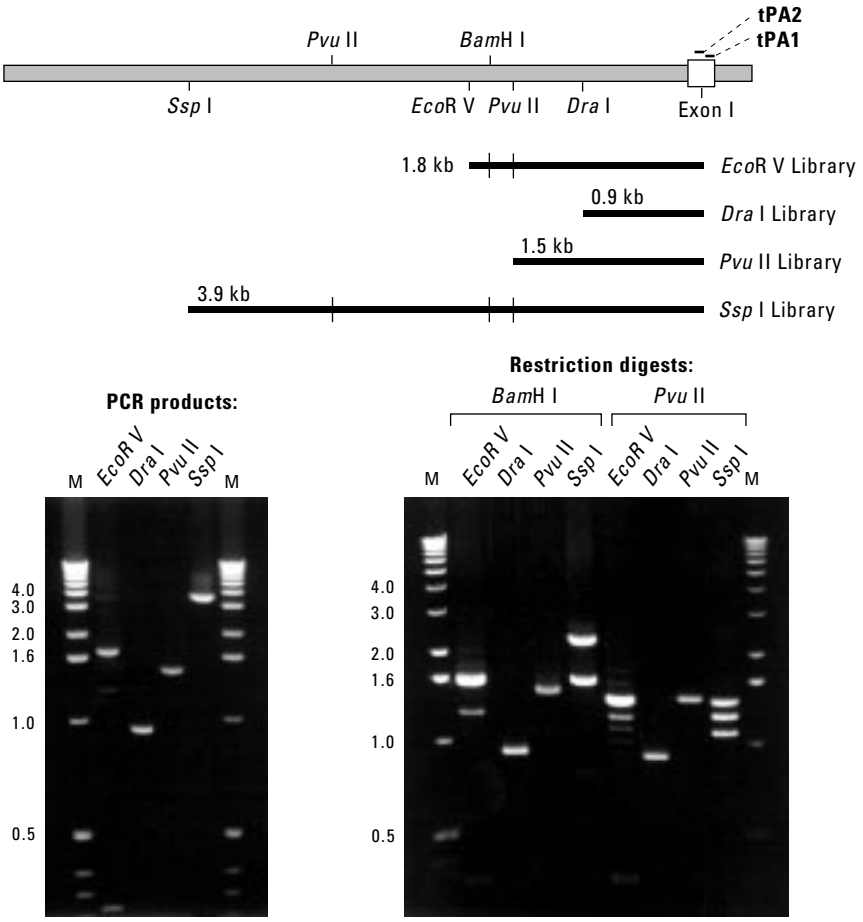

Figure 4. Simple restriction mapping of BD GenomeWalker™ PCR products from the human tPA locus. The map shows the positions of the relevant restriction sites in the genomic DNA and in the predicted BD GenomeWalker PCR products. The gel on the left shows the products of BD GenomeWalker PCR. The gel on the right shows the pattern of restriction fragments generated by digestions of each PCR product with either *Bam*H I or *Pvu* II. Lane M: DNA size markers.

VII. Suggestions for Characterizing Products *continued*

B. Cloning BD GenomeWalker™ Products and Testing for Promoter Activity

1. Cloning BD GenomeWalker products

   Once you have obtained major bands using your gene-specific primer, you will usually want to clone the fragments into a general purpose cloning vector using restriction sites, or into a TA-type cloning vector using the A overhang left by *Taq* DNA polymerase. In some cases, you may wish to clone directly into a promoter reporter vector (See Section B.3 below).

   If your secondary PCR produces a single, major band with little background and no minor bands, you may be able to clone the fragment directly. If the product of your secondary reaction has significant background, you will need to gel-purify the desired band. We recommend either the NucleoSpin® Extract Kit (Cat. No. 635960 or 635961) or the NucleoTrap® Gel Extraction Kit (Cat. No. 636018) for gel-purifying PCR products.

   Note on TAE vs. TBE gels: We recommend that you use Tris-Acetate-EDTA (TAE) buffer instead of Tris-Borate-EDTA (TBE) buffer in your agarose gels when purifying DNA fragments for cloning. In our experience, DNA purified from TBE gels is more difficult to clone than DNA purified from TAE gels.

   Note on EtBr and UV damage to DNA: Minimize the exposure of your DNA to UV light.

2. Sequencing and scanning for regulatory elements

   Prior to testing BD GenomeWalker products for promoter activity, most researchers will want to sequence at least part of their clones and look for common regulatory sequence motifs such as promoters or enhancers.

3. Testing for promoter activity

   BD GenomeWalker products can be cloned into a promoter reporter vector to test for the presence of a promoter. Cloning in both orientations will provide a positive and negative control. Suitable promoter-cloning vectors from BD Biosciences Clontech include the following:

   - **pSEAP2-Basic** is sold separately (Cat. No. 631715) and as a component in the chemiluminescent BD Great EscAPe™ SEAP Reporter System 3 (Cat. No. 631706; Yang *et al*, 1994). This kit also includes pSEAP2-Control and reagents necessary for 100 chemiluminescent assays. The reporter molecule in the BD Great EscAPe system is a secreted form of alkaline phosphatase (SEAP), which can be conveniently measured directly in the culture medium using a sensitive chemiluminescent assay.

VII. Suggestions for Characterizing Products *continued*

- **pβgal-Basic** is sold separately (Cat. No. 631707) and as a component in the Luminescent β-gal Reporter System 3 (Cat. No. 631713; Sinai *et al.*, 1994). This kit also includes pβgal-Control Vector and reagents necessary for 100 chemiluminescent assays.
- **pEGFP-1 Promoter Reporter Vector** (Cat. No. 632319) uses a bright, codon-optimized variant of the green fluorescent protein (GFP) to monitor promoter activity (Cormack *et al.*, 1996; April 1996 *Clontechniques*; Kitts *et al.,* 1995).

All of these vectors have large multiple cloning sites to facilitate cloning.

Note on ATG start codon: If your gene-specific primer was downstream of the ATG start codon in your gene of interest, then you may wish to eliminate the ATG from your promoter reporter construct(s). This may prevent a possible false negative result due to the expression of a bicistronic message (See Section 4.b.v below).

4. Explanation of possible results of tests for promoter activity

   Some BD GenomeWalker products will have no promoter activity when cloned in both orientations in a promoter reporter vector. There are several possible explanations.

   a. None of the fragments contains the promoter.

      Your primer may be several kb from the promoter and/or there may be intervening restriction sites between the primer and the promoter. This may also be an indication that the primer does not fall within the first exon (or within a downstream exon that is within 6 kb of the promoter).

      If this is the case, you may need to obtain sequence data from closer to the 5' end of the transcript. Alternatively, you can “walk another step” by sequencing the distal end of the BD GenomeWalker product(s), designing a new gene-specific primer, and repeating the amplification protocol.

   b. The promoter is present, but the reporter is not expressed.

      There are several possible reasons why you might not detect promoter activity even if your promoter-reporter construct contains the promoter. These include the following:

      i. The fragment is cloned in the wrong orientation.

      Reclone and test in the opposite orientation.

      ii. The promoter is too weak to be detected in your assay.

      If this is the case, it may be possible to add an enhancer to your construct or reclone your fragment(s) in a vector that has an enhancer.

VII. Suggestions for Characterizing Products *continued*

iii. The promoter needs to be induced (and you do not have the means to induce it).

Again, recloning into a vector that has a strong enhancer may allow you to detect promoter activity.

iv. The promoter is tissue- or stage-specific.

Again, recloning into a vector that has a strong enhancer may allow you to detect promoter activity. Alternatively, it may be possible to demonstrate the presence of a promoter by testing the construct in another host cell or in the whole organism.

v. Reporter construct makes a bicistronic message.

The cloned fragment contains the ATG and some portion of the open reading frame from the gene of interest. This results in a bicistronic message in which two ORFs may compete for translation; the downstream ORF (i.e., the reporter) may not be efficiently translated.

If you suspect this to be the case, test for promoter activity at the RNA level by performing RT-PCR. (Reporter expression can be assayed by Northern blot; however, RT-PCR is much faster and more sensitive if suitable primers are available.)

vi. The cloned fragment(s) contains a strong negative enhancer.

There are numerous instances of so-called “negative enhancers” that prevent transcription of a functional promoter. If you suspect this to be the case, try recloning in the presence of a known strong enhancer, or testing subclones in which upstream sequences have been deleted.

5. Deletion analysis of promoters

After finding fragments that have promoter activity, you may want to perform a deletion analysis to define the minimal promoter. Any standard nested-deletion method is compatible with this system.

C. Other Applications of the BD GenomeWalker Method

Other possible applications of the BD GenomeWalker DNA walking method include:

- Mapping intron/exon boundaries.
- Walking short distance upstream or downstream in genomic DNA from known sequences (e.g., expressed sequence tags [EST] or other sequence tagged sites [STS]). Although individual steps are limited to ~6 kb, multiple steps can be strung together to create longer walks.
- Walking from 5' or 3' ends generated by RACE using the BD SMART™ RACE cDNA Amplification Kit (Cat. No. 634914).

VIII. References

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IX. Related Products

For a complete listing of all BD Biosciences Clontech products, please **visit www.bdbiosciences.com/clontech**

	Cat. No.
• BD GenomeWalker™ Kits	
Human	638901
Mouse	638902
Rat	638903
• BD Advantage™ 2 PCR Kit	639206 639207
• BD Advantage™ 2 Polymerase Mix	639201
• BD TaqStart™ Antibody	639250
• BD Great EscAPe™ SEAP2 Reporter System 3 (Includes two vectors, listed below)	631706
pSEAP2-Basic Vector	631715
pSEAP2-Control Vector	631717
• Luminescent β-gal Reporter System 3 (Includes two vectors, listed below)	631713
pβgal-Basic Vector	631707
pβgal-Control Vector	631709
• pEGFP-1 Promoter Reporter Vector	632319
• BD SMART RACE™ cDNA Amplification Kit	634914
• BD Clontech PCR-Select™ cDNA Subtraction Kit	637401
• BD Delta™ Differential Display Kit	637405
• NucleoTrap® Gel Extraction Kit	636018
• NucleoSpin® Extract Kit	635960 635961

Appendix A: Sequence of the Positive Control Primers

The positive control primers in the BD GenomeWalker Universal Kit are derived from exon 1 of the tissue-type plasminogen activator (tPA) cDNA.

PCP1 (tPA1):

5'-AGA AAC CCG ACC TAC CAC GGC TTG CTC CTT-3'

PCP2 (tPA2):

5'-CCC TTT CCT CGC AGA GGT TTT CTC TCC AGC-3'

Appendix B: Design of the BD GenomeWalker™ Adaptor

The BD GenomeWalker Adaptor has three design features that are critical to the success of BD GenomeWalker DNA walking. These features, which can be seen schematically in Figure 1 (in the Introduction), are as follows:

1) The use of a 5'-extended adaptor that has no binding site for the AP1 primer used in primary PCR. An AP1 binding site can only be generated by extension of the gene-specific primer.

2) Blocking of the exposed 3' end of the adaptor with an amine group to prevent extension of the 3' end (which would create an AP1 binding site).

3) The use of an adaptor primer that is shorter than the adaptor itself ("suppression PCR"). As shown in Figure 5, the suppression PCR effect prevents amplification of templates where the 3' end has been extended to create an AP1 binding site. Though rare, such extension does occur, presumably due to incomplete amine modification or incomplete adaptor ligation. Given the exponential nature of PCR amplification, such events would lead to nonspecific amplification and unacceptable backgrounds in the absence of suppression PCR.

Each of these features helps eliminate nonspecific amplification among the general population of DNA fragments. In combination with touchdown PCR and nested PCR, these innovations allow amplification of a specific target from a very complex mixture of DNA fragments—all of which have the same terminal structure—using a single set of gene-specific primers. Of the three features, suppression PCR is the most critical (Siebert *et al.*, 1995).

Appendix B: Design of the BD GenomeWalker™ Adaptor *cont.*

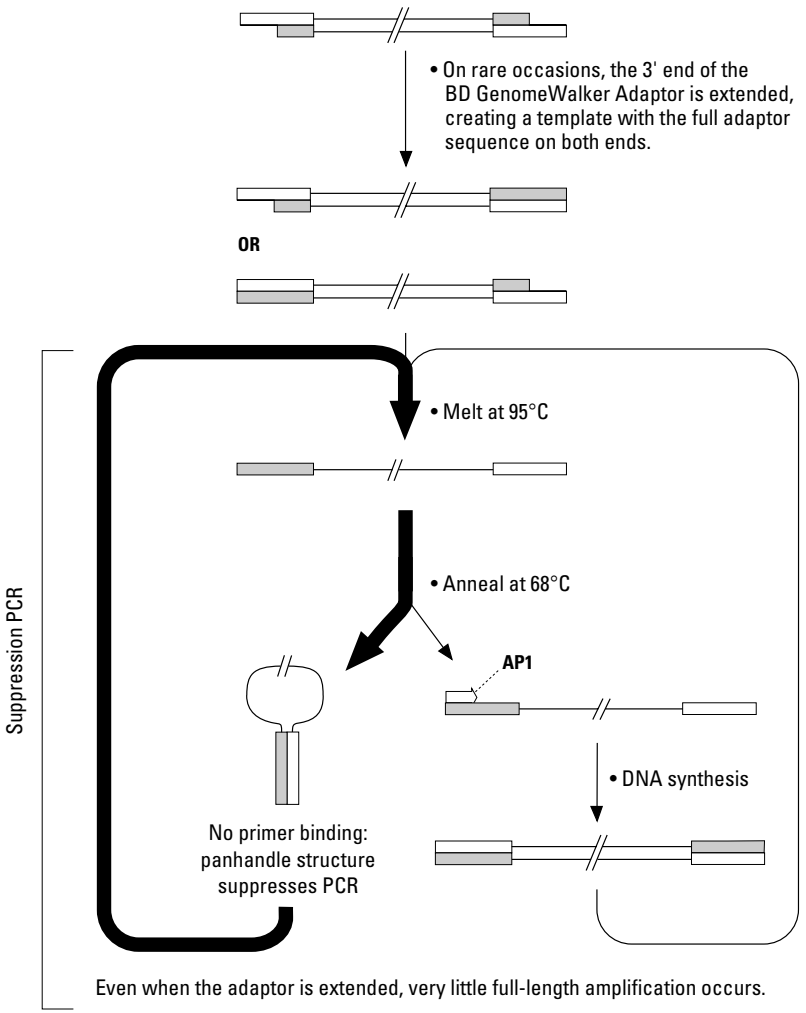
• On rare occasions, the 3' end of the BD GenomeWalker Adaptor is extended, creating a template with the full adaptor sequence on both ends.
OR
• Melt at 95°C
• Anneal at 68°C
AP1
• DNA synthesis
No primer binding: panhandle structure suppresses PCR
Suppression PCR
Even when the adaptor is extended, very little full-length amplification occurs.

Figure 5. The suppression PCR effect. In rare cases, the 3' end of the BD GenomeWalker™ Adaptor gets extended. (Though rare, such extension does occur, presumably due to incomplete amine modification during oligonucleotide synthesis or incomplete adaptor ligation.) This creates a molecule that has the full-length adaptor sequence on both ends and can serve as a template for end-to-end amplification. Without suppression PCR, these rare events would lead to unacceptable backgrounds due to the exponential nature of PCR amplification. However, in suppression PCR, the adaptor primer is much shorter than the adaptor itself. Thus, during subsequent thermal cycling, nearly all the DNA strands will form the "panhandle" structure shown above, which cannot be extended. At the appropriate annealing/extension temperature, this intramolecular annealing event is strongly favored over (and more stable than) the intermolecular annealing of the much shorter adaptor primer to the adaptor. The suppression PCR effect will be reduced or lost if you use an annealing temperature lower than 60–65°C. The upper limit of the suppression PCR effect is about 6 kb.

Appendix C: Parameters for GeneAmp Systems 2400 & 9600

As noted elsewhere in this manual, cycling parameters may have to be optimized for different thermal cyclers. For example, the cycling parameters in this protocol (which were developed on the PE Biosystems DNA Thermal Cycler 480) do not work with their GeneAmp PCR Systems 2400 and 9600. Both the 2400 and 9600 systems use much shorter cycling parameters and smaller thin-walled tubes (0.2-ml vs. 0.5-ml). These systems also eliminate the need to overlay the reaction with mineral oil.

The following parameters for primary and secondary BD GenomeWalker PCR give good results with the standard 50-µl positive control reaction (with no mineral oil overlay) and the 2400 and 9600 thermal cyclers.

Primary PCR (Step V.C.9)

- 7 cycles:

  94°C 2 sec
  72°C 3 min

- 32 cycles:

  94°C 2 sec
  67°C 3 min

- 67°C for an additional 4 min.

Secondary PCR (Step V.C.18)

- 5 cycles:

  94°C 2 sec
  72°C 3 min

- 20 cycles:

  94°C 2 sec
  67°C 3 min

- 67°C for an additional 4 min.

Notes: **Length of denaturation time:** We have observed that differences of only a few seconds in the denaturation time at 94°C can dramatically affect results with the 2400 and 9600 systems. For example, positive control products larger than 2–3 kb were not detectable when the incubation time is increased from 2 to 5 sec. The extremely short incubation time at 94°C may be necessary to preserve the integrity of the larger genomic DNA templates required for LD PCR in the BD GenomeWalker protocol.

Reaction volume: Although the GeneAmp PCR Systems 2400 and 9600 allow you to reduce the reaction volumes in many applications, we have not optimized the BD GenomeWalker protocol for lower reaction volumes.

Notes

Notice to Purchaser

This product is intended to be used for research purposes only. It is not to be used for drug or diagnostic purposes nor is it intended for human use. BD Biosciences Clontech products may not be resold, modified for resale, or used to manufacture commercial products without written approval of BD Biosciences Clontech.

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Suppression PCR is covered by U.S. Patent No. 5,565,340. Foreign patents pending.

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